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Micelles derivatized with octreotide as potential target-selective contrast agents in MRI‡§

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New amphiphilic monomers (OCA-DTPAGlu and OCA-DOTA) containing, in the same molecule, three different functions: (i) the chelating agent (DTPAGlu or DOTA) able to coordinate gadolinium ion, (ii) the octreotide bioactive peptide able to target somatostatin receptors, and (iii) a hydrophobic moiety with two 18-carbon atoms alkyl chains have been designed and synthesized by solid-phase methods. The novel amphiphilic monomers aggregate, in water solution, giving stable micelles at very low concentration (cmc values of 2.3 × 10^{-6} mol kg^{-1} and 2.5 × 10^{-6} mol kg^{-1} for OCA-DTPAGlu and OCA-DOTA, respectively) as confirmed by fluorescence spectroscopy. Fluorescence studies and circular dichroism experiments indicate, for the two compounds as well as for their gadolinium complexes (OCA-DOTA(Gd) and OCA-DTPAGlu(Gd)), the complete exposure of octreotide on the micelle surface, and the predominant presence of an antiparallel β-sheet peptide conformation characterized by a β-like turn. The high relaxivity value (r_{1p} = 13.9 mM^{-1} s^{-1} at 20 MHz and 25 °C), measured for micelles obtained by the gadolinium complex OCA-DTPAGlu(Gd), indicates these aggregates as promising target-selective magnetic resonance imaging (MRI) contrast agents.

Keywords: octreotide; micelles; fluorescence spectroscopy and circular dichroism; MRI contrast agent

Introduction

Somatostatin (SST) is a neuropeptide with a wide role in the physiological control and pharmacological action as inhibitor of growth hormone, insulin and glucagon secretion; its effect is mediated via five G protein coupled membrane receptors (SSTR1-5) [1]. The five SST receptors, especially SSTR2, are widely overexpressed in homogenous and heterogeneous manner in tumor tissues such as gastroenteropancreatic neuroendocrine tumors, gliomas, meningiomas and breast tumors [2–4]. This is a challenging issue in oncology allowing the use of regulatory SST peptide to recognize receptors overexpressed in tumor cells. Unfortunately, SST half-life in plasma is extremely short [5] and the availability of synthetic analogs became an absolute necessity for any progress in this field. Many efforts were dedicated to synthesize a large variety of analogs and to select low molecular weight peptides stable *in vivo* and able to recognize most of the SSTRs. This research led to a cyclic eight-amino acid peptide (octreotide) (Sandostatin) [6] (Figure 1) able to bind to the most frequently overexpressed SSTR2 and to a lesser extent to SSTR5 [7]. For the presence of unnatural D aminoacid residues and the alcoholic *C*-terminus, this peptide is highly resistant to enzymatic degradation and is able to restore the β-turn conformation unavoidable to interact with the receptors [8]. In light of these properties and of nontoxic side effects, octreotide is clearly a useful tool in the cancer management and more than 10 years ago was introduced in clinical practice to limit tumor growth [9]. Several preclinical studies strongly support the concept that binding of octreotide to SSTR is followed by rapid internalization of the receptor–ligand complex [10]. The mechanism of endocytosis is exploited to transport in the cell octreotide-conjugates containing therapeutics and diagnostics, such as the spindle poison taxol [11], and PNA sequences as antisense therapeutics [12]. Moreover, for diagnostic use, several octreotide derivatives, modified on the *N*-terminal moiety with a bifunctional chelator suitable for labeling radiometals such as ^{99m}Tc, ^{111}In, $^{67/68}$Ga, have been designed, synthesized, and used as *in vivo* contrast agents in nuclear medicine techniques positron (emission tomography (PET) and single photon emission computed tomography (SPECT)) [13–16].

These results suggest the application of octreotide as the vehicle of contrast agents in magnetic resonance imaging (MRI). MRI is one of the most efficient *in vivo* imaging techniques, giving very resolved images with anatomical information. In these diagnostic procedures, the images are obtained by accumulating contrast agents. In SPECT or PET, the request concentration of radionuclides in the cancer tissues is 10^{-10} M, while MRI technique, due to its lower sensitivity, needs high concentration (at least 10^{-4} M) of

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§ *Dedicated to Prof. Carlo Pedone on the occasion of his 70th birthday.*

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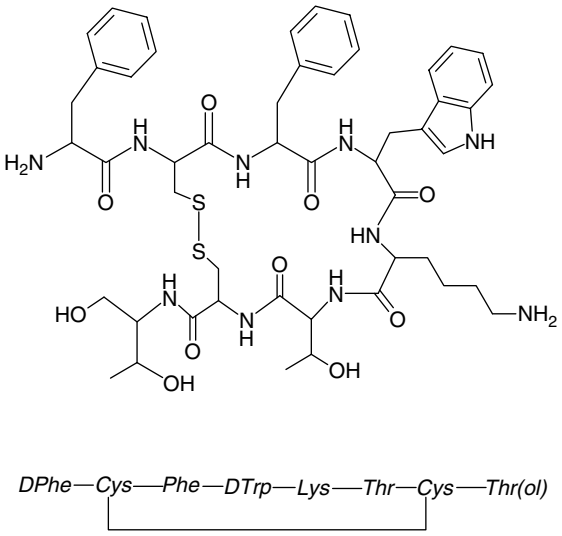

Figure 1. Amino acid sequence and structure of octreotide peptide (Sandostatin).

paramagnetic gadolinium complexes. This critical point can be overtaken by accumulating high concentration of contrast media in the site of interest. The use of a peptide probe based on only one unit of gadolinium complex for a peptide molecule could not overcome the problem of concentration of the contrast agent: most of tumor cells overexpress on their membrane approximately some million of SSTRs leading a number of contrast agents too low in the cells.

To increase the number of contrast agents on the target cells, the binding of a large number of gadolinium complexes to the peptide probe is required. During last years, supramolecular aggregates, such as micelles [17], vesicles and liposomes [18], all containing a high number of stable Gd(III) complexes were prepared. Moreover, supramolecular aggregates derivatized on their external surface with bioactive molecules (peptides or antibodies) have been proposed as target-selective contrast agents in MRI [19]. Following this strategy, we developed several supramolecular aggregates by assembling together two amphiphilic monomers, one containing a gadolinium complex and the other the CCK8 octapeptide able to recognize cholecistokinin receptors overexpressed in human tumor cells [20,21].

More recently we reported the synthesis, the aggregation behavior, the structural characterization and the relaxometric properties of a monomer with an 'upsilon' shape (*MonY*) [22]. The core of this molecule is a lysine residue in which the three reactive functions are derivatized with: the chelating agent *N*,*N*-bis[2-[bis(carboxy-ethyl)amino]ethyl]-L-glutamic acid (DTPAGlu), a DTPA derivative able to give very stable Gd(III) complex; the bioactive *C*-terminal octapeptide sequence of the cholecystokinin hormone (CCK8); and a hydrophobic moiety containing two alkyl chains with 18 carbon atoms each. In this paper we report, the synthesis in solid phase, the aggregation properties and the relaxometric behavior of two novel octreotride-conjugate amphiphiles (OCA: OCA-DOTA and OCA-DTPAGlu) in which the hydrophobic moiety is formed by two hydrocarbon tails at 18 carbon atoms each, while the hydrophilic head group still contains the DTPAGlu or tetraazacyclododecane-tetraacetic acid (DOTA) chelating agents or their gadolinium complexes, and the octreotide bioactive peptide (Figure 2).

Results and Discussion

Monomers Design and Synthesis

The monomers design has to fulfil two requirements: (i) chemical modifications on octreotide should preserve the peptide receptor binding capability; (ii) after the aggregation process, the peptide molecules and the gadolinium complexes have to lie in hydrophilic shell of the supramolecular aggregates to preserve the peptide bioactivity and to allow water exchange in the gadolinium coordination sphere. The first requirement was fulfilled by introducing modifications at *N*-terminal end of the peptide sequence. In fact, recent studies show that a hydrophilic or hydrophobic chain covalently bound on the *N*-terminus of DPhe residue results in low effect on binding constants of octreotide to the receptors [23]. The second need could be satisfied by introducing two units of 21-amino-4,7,10,13,16,19-hexaoxaheneicosanoic acid (AhOH) between the lysine residue and the octreotide *N*-terminal residue. The AhOH linker was chosen to increase the hydrophilicity of the head without change the charge of the monomers. Moreover, the oxoethylene moieties are not toxic, are able to reduce aggregate clearance through the reticulo-endothelial system (RES) [24] and they avoid adsorption of blood proteins into the supramolecular aggregates.

The length of linkers was selected, according to the biological results previously obtained on similar aggregates derivatized with CCK8 or 7-14-bombesin, in order to favor a good exposure of octreotide on the external aggregate surface. The branching of the monomers headgroup was achieved using a lysine residue. On its α amine function, another unit of the linker was bound to space the hydrophilic head from the lipophilic tails; while on lysine side chain, the selected DOTA or DTPAGlu chelating agents were condensed to distance them far away from the octreotide moiety.

DOTA belongs to the macrocycle class of chelating agents while DPTAGlu is a branched chelating agent. Both ligands are able to coordinate Gd(III) ions with high-stability constants saturating eight coordination positions, thus leaving the last position for water coordination and allowing water exchange in gadolinium coordination sphere. They were selected to study the influence of the steric hindrance and of the residual charge present in the hydrophilic shell on the aggregation behavior.

The chemical synthesis of the two monomers, OCA-DOTA and OCA-DTPAGlu, was fully performed on solid support according to standard SPPS protocols following the Fmoc/*t*Bu chemistry [25]. The synthetic scheme is reported in detail in Figure 3. The preloaded H-Thr(*t*Bu)-ol-(2-chrolo-trityl) polymeric resin was used to obtain the alcoholic function on the *C*-terminus. The asymmetric compounds were obtained by the introduction of a lysine residue, orthogonally protected by Dde and Fmoc on the α and ε amine functions, respectively. The Dde protecting group was preferred to Mtt protecting group to avoid a partial cleavage from the resin in acid-labile removal conditions and because of its stability during the Fmoc removal by treatment with piperidine. After removal of Fmoc, DTPAGlu-pentaester or DOTA-triester, activated by HATU, were condensed on lysine side chain. The Dde removal was obtained by brief treatment with 2% hydrazine in *N*,*N*-dimethylformamide, giving the α free amine function, on which the AhOH linker and *N*,*N*-dioctadecylsuccinamic acid were condensed. At the end of the monomers assembly, acetamidomethyl (Acm) removal from the cysteine residues and disulfide bridge formation were achieved by $Tl(CF_3CO_2)_3$ treatment on a solid support. Cleavage was performed using standard trifluoroacetic acid (TFA)/tri-isopropylsilane (TIS)/H_2O mixtures, to allow complete removal of protecting groups from all the amino acid side chains

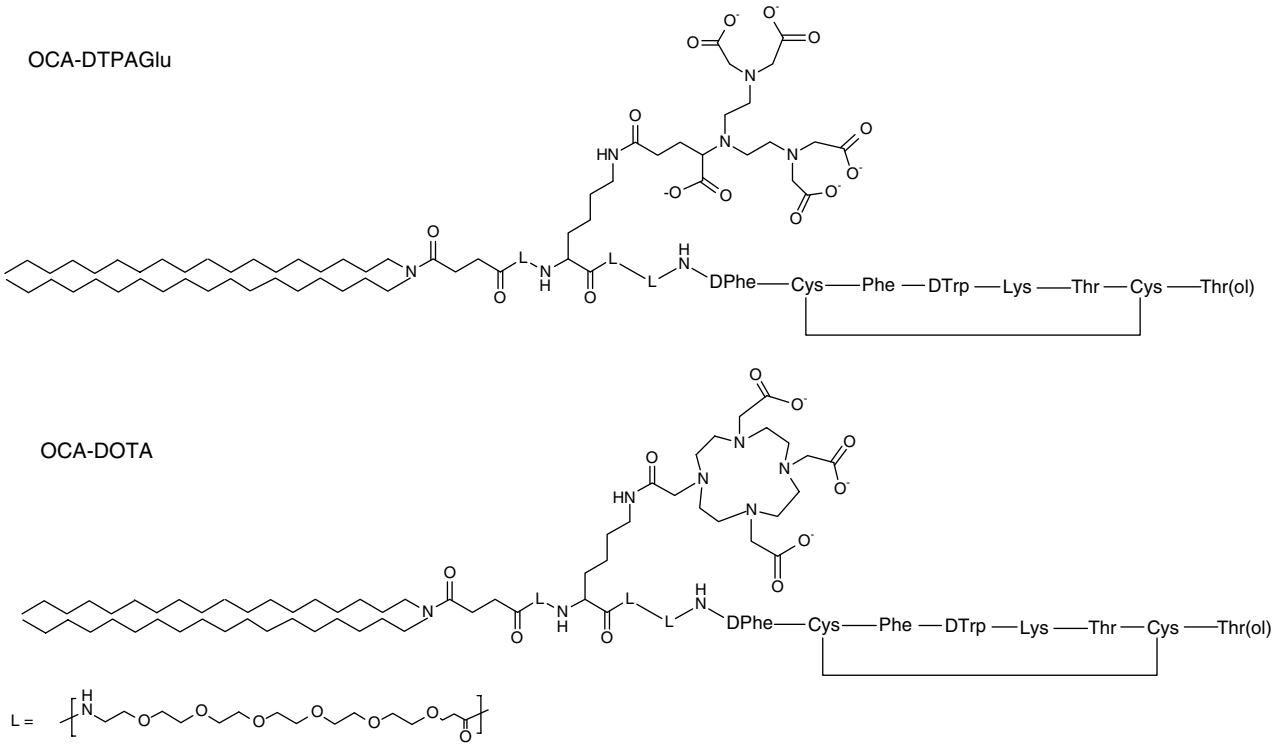

Figure 2. Schematic representation of the two octreotide conjugate amphiphiles: $(C18)_2$-(AhOH)-Lys(DTPAGlu)-$(AhOH)_2$-octreotide, OCA-DTPAGlu, and $(C18)_2$-(AhOH)-Lys(DOTA)-$(AhOH)_2$-octreotide, OCA-DOTA.

and from chelating agents carboxylic groups. The crude products were purified by preparative reversed-phase HPLC to a final purity of 90% and isolated in 30–40% yields in lyophilized form. Their molecular masses were determined by MALDI-TOF mass spectroscopy.

The OCA-DOTA(Gd) and OCA-DTPAGlu(Gd) gadolinium complexes were obtained by adding increasing amounts of a concentrated $GdCl_3$ solution to the free base monomers at neutral pH and at room temperature. To avoid the relaxivity contribution of free gadolinium, the excess of ions was removed at pH 10 as insoluble hydroxide as already reported for other DTPAGlu or DOTA gadolinium complexes. The Gd(III) titration is conveniently followed by measuring ^{1}H-relaxation rates.

Aggregates Preparation and Characterization

Supramolecular aggregates have been prepared by dissolving OCA-DOTA and OCA-DTPAGlu free base or their corresponding gadolinium complexes (OCA-DOTA(Gd) and OCA-DTPAGlu(Gd)), in phosphate buffer at pH 7.4 following well-assessed procedures.

On the basis of structural characterizations performed on self-assembling aggregates of a similar branched amphiphilic compound containing the CCK8 peptide and the DTPAGlu chelating agent, small micelles are expected for OCA-DOTA, OCA-DTPAGlu and their gadolinium complexes, in water solution [22]. Anyway, the self assembling of the amphiphilic compounds occurs only over a critical micellar concentration (cmc) that can be measured by a fluorescence based method using 8-anilino-1-napthalene sulfonic acid ammonium salt (ANS) as a probe. This aromatic organic molecule is fluorescence sensitive according to the environment polarity: it is essentially nonfluorescent in water, but highly fluorescent in nonpolar environments such as the inner micellar compartment [26]. The wavelength of maximum fluorescence intensity varies with the environment, and usually occurs between 450 and 480 nm. The addition of the amphiphilic surfactant to the aqueous ANS solution causes an increase in the fluorescence intensity which reaches a *plateau* at higher concentrations, when all the dye is entrapped in the micellar hydrophobic compartment. The experimental measure of the ANS fluorescence intensity at 480 nm, corresponding to the maximum of spectrum, on function of OCA-DOTA or OCA-DTPAGlu concentration (Figure 4(a)), provides the cmc values. The break points in Figure 4(a) indicate cmc values of 2.3×10^{-6} mol kg^{-1} and 2.5×10^{-6} mol kg^{-1} for OCA-DTPAGlu and OCA-DOTA, respectively, thus confirming the expected high stability of the resulting supramolecular aggregates. The same experiments produce the plot reported in Figure 4(b) for the corresponding gadolinium complexes OCA-DTPAGlu(Gd) and OCA-DOTA(Gd). The first monomer aggregates at concentrations higher than 2.0×10^{-5} mol kg^{-1}, while the second one does not give supramolecular structures at concentrations lower than 2.6×10^{-4} mol kg^{-1}, as clearly indicated by the fluorescence experiment. In fact, ANS probe remains not fluorescent during the titration with the amphiphilic molecule, thus indicating that an aggregation process occurs. The different behavior between the two monomers could be explained as a function of the different number of negative charges preserved on the head group after the gadolinium complexation: OCA-DTPAGlu(Gd) monomer retains two negative charges, while OCA-DOTA has no charges. The complete loss of charges on the Gd-DOTA gadolinium complex produces a remarkable reduction of the anionic character of the amphiphilic monomer which is unable to aggregate [27].

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Figure 3. Scheme of the chemical procedures for the solid-phase synthesis of the two monomers OCA-DTPAGlu and OCA-DOTA. Resin is schematically represented as a broken curve.

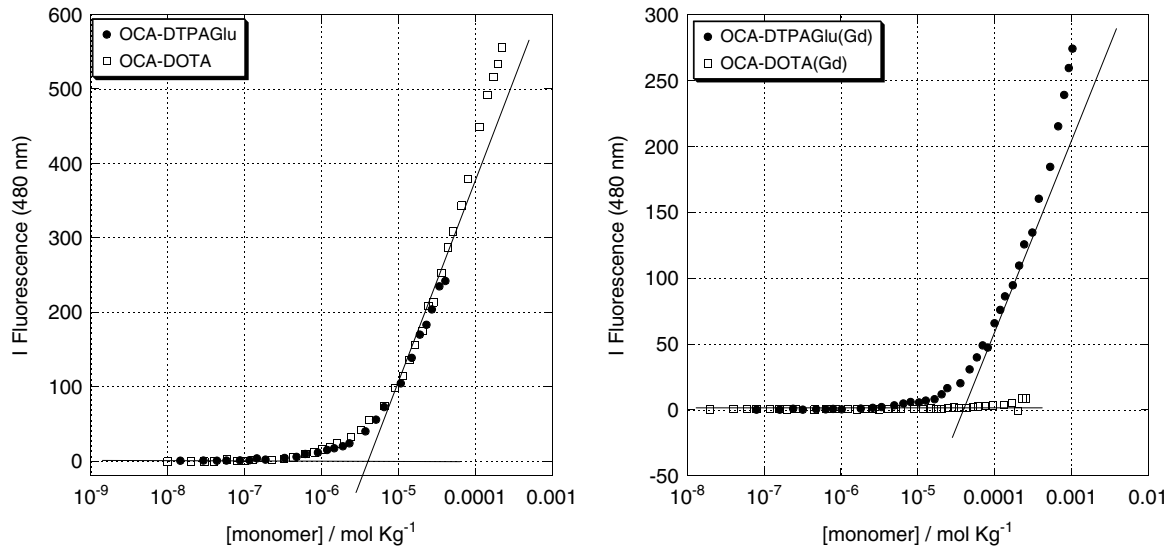

Figure 4. Fluorescence intensity of ANS fluorophore at 480 nm *versus*: (a) OCA-DTPAGlu and OCA-DOTA concentration; (b) OCA-DTPAGlu(Gd) and OCA-DOTA(Gd) concentration. The cmc values are established from graphical break points.

Physicochemical Studies on the Octreotide Moiety

Biological data concerning SST analogs obtained by introducing covalent conformational constraints, suggested that -Phe-D-Trp-Lys-Thr- amino acidic sequence contains all the elements necessary for the expression of the SST biological activities *versus* SSTR2 [7]. Literature NMR studies indicated that octreotide adopts a predominant antiparallel β-sheet conformation characterized by a type II′ β-like turn across residues D-Trp[4] and Lys[5] [28], and these results were also confirmed by X ray structural studies [29]. Therefore, all octreotide conjugates should preserve this conformation and chemical alterations should not affect the D-Trp residue. Fluorescence experiments and CD studies were

carried out to prove that the peptide moiety in the amphiphilic molecules keeps the conformational requirements unaltered also after micelles formation and that it remains well exposed on the micelle surface. The exposure of the bioactive portion of the monomers on aggregates surface was assessed by monitoring the fluorescence emission due to the indole moiety of the tryptophan residue. Usually, this fluorophore shows an emission peak centered at 350 nm in polar solvents while in hydrophobic solvents the maximum shifts occurs at 330 nm [30]. The fluorescence emission spectra of the three aggregates (Figure 5) were recorded at 25 °C and at monomer concentration (1.0×10^{-5} M for OCA-DOTA and OCA-DTPAGlu; 1.0×10^{-4} for OCA-DTPAGlu(Gd)), higher than cmc to be sure of the presence of aggregates in solution. The fluorescence maximum at 350 nm suggests for OCA-DOTA, a complete exposure of D-Trp residue in the hydrophilic external space. In the case of OCA-DTPAGlu and OCA-DTPAGlu(Gd), the maximum is centered at 346 nm, this value, slightly shifted with respect to 350 nm, indicates that most of the indole groups on tryptophan side-chain lies in the hydrophilic environment. The slight difference between OCA-DOTA and OCA-DTPAGlu could be attribute to the different steric hindrance of the chelating agents which can drive the micelle structure. Instead, the octreotide disposition seems not to be affected by gadolinium coordination, reducing the negative charge on the hydrophilic surface. This result suggests the use of the octreotide peptide to drive the entire aggregate toward SST receptors present on cell membranes.

The effects on the conformational structure of the octreotide peptide in the two amphiphilic molecules were assayed by CD measurements. Figure 6(a) shows the CD spectra of OCA-DOTA and OCA-DTPAGlu at 1.0×10^{-4} M concentration, above their cmc values. The octreotide wild-type spectrum was also reported for comparison. All spectra indicate predominant presence of an antiparallel β-sheet conformation characterized by a β-like turn, clearly suggesting that the disulphide bridge is conserved on the aggregates surface. OCA-DTPAGlu and octreotide spectra are nearly superimposable in the 190–260 nm range. Instead, the OCA-DOTA spectrum in 190–210 nm range shows a blue shift of the minimum, which may indicate some conformational modifications, but widely confirms the β-sheet conformation.

Moreover, OCA-DTPAGlu spectra at three different concentrations are also reported in Figure 6(b). The presence of oxoethylenic linkers and lipophilic tails on peptide *N*-terminus could induce some changes in peptide conformation. By decreasing concentration below the cmc, even if some spectral modifications are observed, the β-sheet rearrangement is preserved. This variation disappears when the monomer self-aggregates in solution above cmc. The steric hindrance on micelles surface might reduce the degree of freedom and restore the minimum octreotide conformation.

Relaxivity Measurements

The parameter for an evaluation of an MRI contrast agent is its relaxivity, i.e. the potency to shorten the relaxation times of the solvent water protons. The measured relaxivity value (r_{1p}) is defined as the paramagnetic contribution to the measured proton longitudinal relaxation rate (R_{1obs}) of a solution containing 1.0 mM concentration of gadolinium according to Eqn (1) [31]

$$R_{1obs} = [Gd] \cdot r_{1p} + R_{1W} \quad (1)$$

where R_{1w} is the diamagnetic contribution of pure water (0.38 s^{-1}).

The relaxivity of OCA-DTPAGlu(Gd), was measured at monomer concentration higher than the calculated cmc to be sure that the molecules are aggregated in the micellar form. A value of 13.9 mM^{-1} s^{-1} has been determined at 20 MHz and 25 °C (Figure 7). This value was then confirmed mineralizing, with HCl 37% at 120 °C overnight, a given quantity of sample solution to determine the exact concentration of Gd(III) present in the solution: from the measure of the observed relaxation rate (R_{1obs}) of the acidic solution, knowing the relaxivity (r_{1p}) of Gd(III) aquoion in acidic conditions (13.5 mM^{-1} s^{-1}), it was possible to calculate the exact Gd(III) concentration (Eqn (1)) (This method was calibrated using standard inductively coupled plasma (ICP) solutions, and the accuracy was determined to be 1%). At this point, knowing [GdL] and measuring R_{1obs} of the micellar mother solution, the same Eqn (1) was used to calculate the micellar relaxivity. Relaxivity value of OCA-DTPAGlu(Gd) micelles (13.9 mM^{-1} s^{-1}) is in the same range of the value previously reported (15.0 mM^{-1} s^{-1}) for aggregates obtained by self assembling of a similar monomer containing the CCK8 peptide (*MonY*-Gd) [22]. The slight difference could be probably ascribed to different aggregation number or size.

The analysis of nuclear magnetic relaxation dispersion (NMRD) profile of the lipophilic aggregated system has been made according to the Solomon–Bloembergen–Morgan model, modified

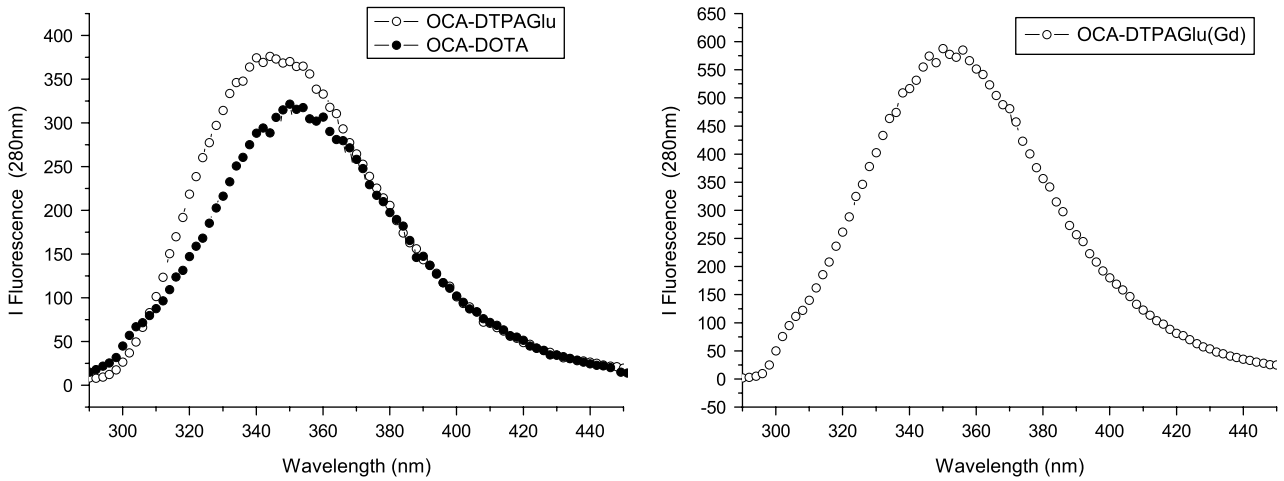

Figure 5. Fluorescence spectrum of tryptophan residue of: (a) OCA-DTPAGlu and OCA-DOTA aggregates; (b) OCA-DTPAGlu(Gd) aggregate. Fluorescence spectra were excited at 280 nm.

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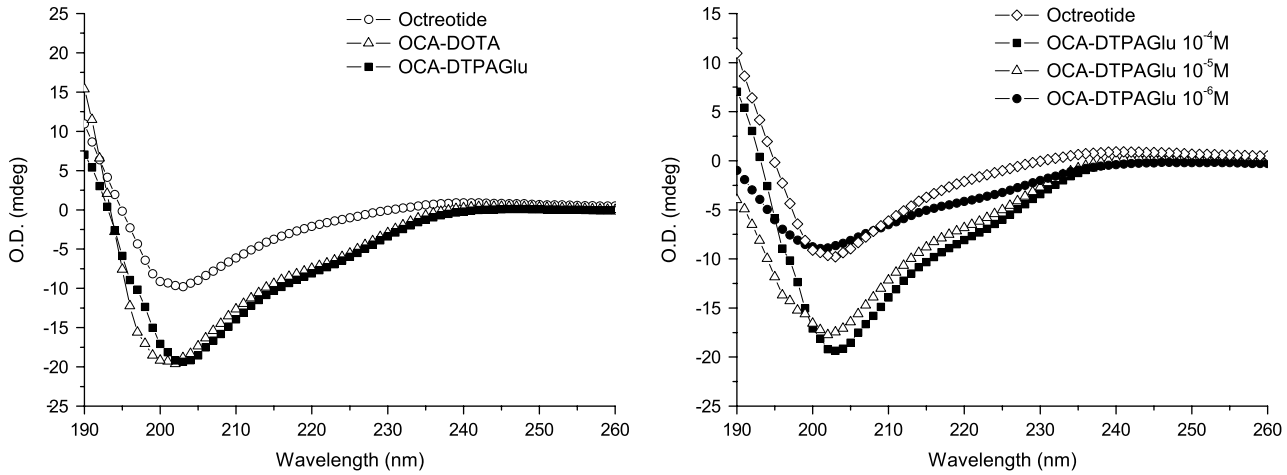

Figure 6. Circular dichroism profiles of: (a) octreotide, OCA-DTPAGlu and OCA-DOTA (b) of octreotide and OCA-DTPAGlu at 1.0×10^{-4} M, 1.0×10^{-5} M, 1.0×10^{-6} M concentrations.

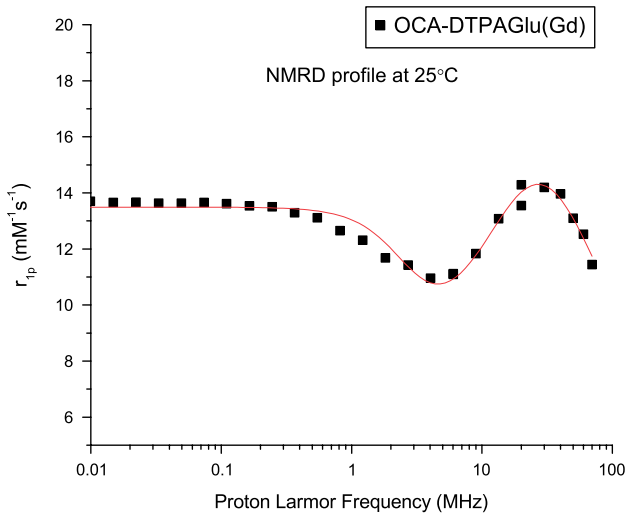

Figure 7. 1/T1 NMRD profile of OCA-DTPAGlu(Gd) at pH 7.4 and 25 °C, normalized to 1 mM Gd (III) ion. This figure is available in colour online at www.interscience.wiley.com/journal/jpepsci.

according to the Lipari-Szabo approach [32–34] to obtain an accurate determination of the reorientational correlation time (τ_R), that is strictly related to the molecular size of the investigated system. This model is generally applied to the systems with a faster local motion (governed by τ_l) and a slower global motion (governed by τ_g); the extent of local to global contribution to the overall motion is determined by an order parameter (S^2) that can vary from 0 to 1.

The experimental data $\tau_l = 250$ ps, $\tau_g = 2900$ ps and $S = 0.3$ were fitted by considering one water molecule in the inner coordination sphere for each Gd(III) complex ($n_w = 1$) and fixing the exchange lifetime of the coordinated water molecule (τ_M) to the (60 ns) previously determined for the similar micellar system containing the CCK8-peptide functionalized monomer (*MonY*-Gd) [22] because of the low solubility of OCA-DTPAGlu(Gd) system, which prevents any direct ^{17}O-NMR R_{2p} *versus* T measurement.

In Vitro Biological Assays

Receptor binding ability of OCA-DTPAGlu in micellar form has been evaluated by standard nuclear medicine experiments. Radiolabeling of the aggregates was performed at concentrations above critical micellar concentration to avoid the presence of free monomers in solution. Trace amounts of $^{111}InCl_3$ and up to 500 μCi were added to the aggregate formulation after addition of an equal volume of 0.5 N sodium citrate. Confirmation of incorporation of the radioactive label into the aggregates was obtained by gel filtration. ^{111}In-labeled aggregates showed preferential binding to A431 cells overexpressing the sstr2 by transfection compared to control cells; in fact 3.8% of bound plus internalized radioactivity in receptor expressing cell against 1.6% for control cells was obtained in a preliminary experiment performed at 37 °C.

Conclusions

New amphiphilic monomers (OCA-DTPAGlu and OCA-DOTA) containing, in the same molecule, three different functions: (i) the chelating agent (DTPAGlu or DOTA) able to coordinate gadolinium ion, (ii) the octreotide bioactive peptide able to target SSTR2 and SSTR5 SST receptors and (iii) a hydrophobic moiety with two 18-carbon atoms alkyl chains, have been designed and synthesized by solid-phase methods. In water solution at pH 7.4, the peptide-conjugate amphiphiles self-assemble as micelles at low concentration as confirmed by fluorescence measurements, using the fluorescent ANS probe. The corresponding gadolinium complexes present a different aggregation behavior: While OCA-DTPAGlu(Gd) gives stable micelles, OCA-DOTA(Gd), which does not contain charges on the hydrophilic head, remains in the nonaggregated state in the explored concentration range. As consequence of this aggregation behavior, OCA-DTPAGlu(Gd) seems a very promising target-selective MRI contrast agent. It presents the high relaxivity value expected for amphiphilic gadolinium complexes in aggregated form; a high stability of the micelles, and above all the exposure of the bioactive octreotide peptide on the micelle external surface, in the correct conformation to bind the target receptors. The selective binding of OCA-DTPAGlu in micellar form was determined in a preliminary experiment, in which the octreotide derivative, at concentration higher than its cmc, is labeled with ^{111}In. ^{111}In-labeled aggregates show preferential binding to A431 cells overexpressing SSTR2 compared to control cells (3.8% against 1.6% of bound plus internalized radioactivity). As already reported in the case of

a similar compound containing the CCK8 peptide, *Mon*Y-Gd, micelles of OCA-DTPAGlu(Gd) belongs to novel class of MRI contrast agents. They could be able to target SSTR2 and SSTR5 receptors, well-assessed targets for several human tumors.

Experimental Methods

Materials and Methods

Protected N^{α}-Fmoc-amino acid derivatives, coupling reagents and H-Thr(*t*Bu)-ol-(2-chrolo-trityl)-resin were obtained from Calbiochem-Novabiochem (Laufelfingen, Switzerland) and INBIOS (Napoli, Italy). Fmoc-21-amino-4,7,10,13,16,19-hexaoxaheneicosanoic acid (Fmoc-AhOH-OH) was purchased from Neosystem (Strasbourg, France). The 1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetate terbutil ester [(DOTA(tBu)$_3$] was purchased from Macrocycles (Richardson, Texas). DTPAGlu pentaester chelating agent (*N*2,*N*2-bis[2-[bis[2-(1,1-dimethylethoxy)-2-oxoethyl]-amino]ethyl]-L-glutammic 1,1-dimethylethyl ester) and the *N*,*N*-dioctadecylsuccinamic acid were synthesized according to the experimental procedure reported in literature [35,36]. All other chemicals were commercially available at Sigma-Aldrich (Milwaukee WI, USA) or Fluka (Buchs, Switzerland) and were used without further purification. All solutions were prepared by weight using doubly distilled water. The pH of all solutions was kept constant at 7.4 in phosphate buffer.

Synthesis of OCA-DOTA and OCA-DTPAGlu

Peptide synthesis was carried out in solid-phase under standard conditions using Fmoc strategy, [25] on H-Thr(*t*Bu)-ol-(2-chrolo-trityl)-resin (0.70 mmol/g, 0.15 mmol scale, 0.214 g). The peptide chain was built by sequential coupling and Fmoc deprotection of the following seven Fmoc-amino acid derivatives: Fmoc-Cys(Acm)-OH, Fmoc-Thr(O*t*Bu)-OH, Fmoc-Lys(Boc)-OH, Fmoc-DTrp(Boc)-OH, Fmoc-Phe-OH, Fmoc-Cys(Acm)-OH, Fmoc-DPhe-OH. All couplings were performed twice for 60 min, by using an excess of four equivalents for the single amino acid derivative. The carboxylic functions of α-amino acids were activated *in situ* by the standard 1-hydroxybenzotriazole/benzotriazol-1-yl-oxy-tris-pyrrolidino-phosphonium/*N*,*N*-diisopropylethylamine (HOBt/PyBop/DIPEA) procedures in DMF. Fmoc deprotections were obtained by 30% piperidine solution in DMF. The coupling steps were monitored by the qualitative Kaiser test. When the peptide synthesis was complete, the Fmoc *N*-terminal protecting group was removed and two residues of Fmoc-AhOH-OH were condensed by using, for each residue, an excess of two equivalents activating the carboxylic function similarly in a single coupling for 60 min under N_2 flow at room temperature. After Fmoc removal from the *N*-terminal end of the peptide derivative, 0,320 g (0.60 mmol) of Dde-Lys(Fmoc)-OH, activated by a stoichiometric amount of PyBop and HOBt and two equivalents of DIPEA in DMF, were coupled by stirring the slurry suspension of the resin for 60 min. The solution was filtered and the resin was washed with three portions of DMF. After removal of the Fmoc group on side chain of lysine residue, the chelating agent (DTPAGlu pentaester or DOTA trisester) was linked, through its free carboxyl function, to the ε-NH_2 of the lysine residue. This coupling step was performed using two equivalents of the chelating agent, HATU and four equivalents of DIPEA in DMF as a solvent. The coupling time, compared with the classical solid-phase peptide synthesis protocol, was increased up to 120 min. The resin was washed three times with DMF, then, the 1-(4,4-dimethyl-2,6-dioxo-cyclohexylidene)3-methylbutyl group (Dde) was removed by treatment with DMF/Hydrazine mixture (98 : 2). The peptide-resin was stirred with 3.0 ml of this solution for 10 min. The treatment was repeated twice, and the reaction was monitored by the qualitative Kaiser test. On *N*-terminal moiety, the Fmoc-AhOH-OH linker was coupled following the same procedure above described. After the removal of *N*-terminal Fmoc protecting group, *N*,*N*-dioctadecylsuccinamic acid (0.187 g, 0.3 mmol) in DMF/DCM (1/1) mixture were condensed. The coupling reaction was repeated twice under N_2 flow for 60 min. The lipophilic moiety was activated *in situ* by the standard HOBt/PyBop/DIPEA procedure. The deprotection (Acm removal) and the oxidation reaction of cysteine residues were carried out at same time adding 1.2 eq $Tl(CF_3CO_2)_3$ to a suspension of the peptidyl resin in DMF/anisole (19 : 1) stirring the mixture for 18 h at 0 °C monitoring at times by the colorimetric Ellmann test [37].

The peptide derivatives were cleaved from the solid support by suspending the resin in 10 ml of TFA/TIS/H_2O (95.5/2/2.5) mixture for 120 min. During this step all the amino acid side chains and the carboxylic groups of the chelating moiety were simultaneously freed from their protecting groups. Free peptide derivatives were precipitated in cold water and lyophilized from a 50% H_2O/CH_3CN solution. The crude compounds were purified by RP-HPLC. Preparative RP-HPLC was carried out on a Shimadzu 8A (Kyoto, Japan) apparatus equipped with an UV Shimadzu detector using a Phenomenex (Torrance, CA) C4 column, 22 mm × 250 mm with a flow rate of 20 ml min^{-1}. The single peaks were analyzed by HPLC and mass spectrometry. Analytical RP-HPLC runs were carried out on a HP Agilent Series 1100 apparatus (Santa Clara, CA, USA) using a Phenomenex (Torrance, CA) C18 column, 4.6 mm × 250 mm with a flow rate of 1.0 ml min^{-1}. For all the RP-HPLC procedures, the system solvent used was H_2O 0.1% TFA (A) and CH_3CN 0.1% TFA (B), with a linear gradient from from 5 to 70% B in 30 min followed by 70 to 95% B in 10 min. Mass spectral analysis were carried out on MALDI-TOF Voyager-DE mass spectrometer Perseptive Biosystems (Framigham, MA, USA). The desired compounds (∼180 mg) were obtained at HPLC purity higher than 95% with a final yield of around 10%.

$(C18)_2$-(AhOH)-Lys(DTPAGlu)-$(AhOH)_2$-octreotide, OCA-DTPAGlu, *Rt* = 41.0 min; *MW* = 3204 amu.
$(C18)_2$-(AhOH)-Lys(DOTA)-$(AhOH)_2$-octreotide, OCA-DOTA, *Rt* = 41.3 min; *MW* = 3144 amu.

Preparation of Gadolinium Complexes

Gadolinium complexes have been obtained by adding light excess of the $GdCl_3$ to aqueous solution of OCA-DTPAGlu and OCA-DOTA ligands at neutral pH and room temperature. The formation of the gadolinium complexes was followed by measuring the solvent proton relaxation rate (1/T1). The excess of uncomplexed Gd(III) ions, which yields a variation of the observed relaxation rate, was removed by centrifugation of the solution brought to pH 10; further relaxation rate measurements were made to check the complete Gd(III) ions removal.

Preparation of Micelle Containing Solutions

Stock solutions of OCA-DTPAGlu and OCA-DOTA monomers and of their gadolinium complexes were prepared by stirring the monomers until complete dissolution in 0.1 M phosphate buffer at pH 7.4 and filtering through a 0.45 µm filter. Concentrations of all solutions (1.0×10^{-3} M for OCA-DTPAGlu, OCA-DOTA

and OCA-DTPAGlu(Gd); and 1.0×10^{-4} M for OCA-DOTA(Gd)) were determined by absorbance on a UV–vis Jasco V-5505 spectrophotometer (Easton, MD, USA) equipped with a Jasco ETC-505T Peltier temperature controller with a 1-cm quartz cuvette using a molar absorptivity (ε_{280}) of 5630 M^{-1} cm^{-1} for octreotide, due to the contribution of tryptophan residue present in the primary octreotide structure [38,39].

Fluorescence Measurements

Emission spectra were recorded using a Jasco Model FP-750 spectrofluorimeter equipped with a Peltier temperature controller in 1.0-cm path length quartz cell at 25 °C. Equal excitation and emission bandwidths were used throughout experiments, with a recording speed of 125 nm min^{-1} and automatic selection of the time constant. The cmc values were obtained by using 8-anilinonaphthalene-1-sulfonate (ANS) as a fluorescent probe. Small aliquots of 1×10^{-4} M aggregates solution, were added to a fixed volume of fluorophore in the cell (1×10^{-5} M ANS) dissolved in the same buffer. The cmc values were determined by linear least-squares fitting of the fluorescence emission at 480 nm, upon excitation at 350 nm *versus* the amphiphile concentration as previously reported [26,40].

Tryptophan emission spectra in 290–450 nm range were obtained exciting at 280 nm micelle solutions at monomer concentration of 1.0×10^{-5} M for OCA-DTPAGlu and OCA-DOTA; and 1.0×10^{-4} M for OCA-DTPAGlu(Gd) and OCA-DOTA(Gd).

Circular Dichroism (CD) Experiments

Far-UV CD spectra were collected at room temperature on a Jasco J-810 spectropolarimeter equipped with a NesLab RTE111 thermal controller unity using a 1-mm quartz cell. Other experimental settings were: scan speed, 10 nm/min; sensitivity, 50 mdeg; time constant, 16 s; bandwidth, 3 nm. CD measurements were conducted on solutions containing peptide amphiphilic surfactant at concentrations of 1×10^{-4} M, 1×10^{-5} M and 1×10^{-6} M in 2.5 mM phosphate buffer at pH 7.4. The CD spectra were collected from 260 to 190 nm, corrected for blank and adjusted for dilution.

Water Proton Relaxation Measurements

The longitudinal water proton relaxation rates were measured on a Stelar Spinmaster (Mede, Pavia, Italy) spectrometer operating at 20 MHz, by means of the standard inversion-recovery technique (16 experiments, 2 scans). A typical 90° pulse width was 4 μs and the reproducibility of the T_1 data was ±0.5%. The temperature was maintained at 298 K with a Stelar VTC-91 air-flow heater equipped with a copper-constantan thermocouple (uncertainty ±0.1 °C). The proton $1/T_1$ NMRD profiles were measured over a continuum of magnetic field strength from 0.00024 to 0.28 *T* (corresponding to 0.01–20 MHz proton Larmor Frequency) on a Stelar fast field-cycling relaxometer. This relaxometer works under complete computer control with an absolute uncertainty in $1/T_1$ of ±1%. Data points from 0.47 *T* (20 MHz) to 1.7 *T* (70 MHz) were collected on a Stelar Spinmaster spectrometer working at variable field.

Biological Assays

Radiolabeling of OCA-DTPAGlu aggregates was performed at monomer concentration of 1.0×10^{-3} M. Trace amounts of $^{111}InCl_3$ and up to 500 μCi were added to the aggregate formulation after addition of an equal volume of 0.5 N sodium citrate. Confirmation of incorporation of the label into the aggregates was obtained by gel filtration on Sephadex G-50 prepacked columns (Pharmacia Biotech). Binding activity was tested on A431 cells overexpressing the SSTR2 by stable transfection and compared to control cells. Assays were performed at 37 °C on cell suspensions that were incubated with the radiolabel aggregates for 1 h. Concentration of the monomers in the cell binding assays were always kept above 10^{-4} M. Radioactivity bound to cells was separated from unbound activity by centrifugation through dibutyl phthalate in 1.5 ml tubes which were subsequently frozen on dry ice, the cell pellet containing portion of the tube excised and counted in a Wallac gamma counter. Unbound radioactivity was placed in a separate vial and counted as well.

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